Adherence, haemagglutination and cell surface characteristics of motile aeromonads virulent for fish

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Abstract. Motile *Aeromonas* spp. virulent for fish were studied with regard to their adhesion profile. Electron microscopy demonstrated the presence of fimbriae (pili) on *Aeromonas* cells regardless of virulence potential. The results show no significant correlations between ability to haemagglutinate, yeast cell co-agglutination and virulence. All strains expressed mannose-sensitive haemagglutinin activity against guinea pig erythrocytes. Using four types of red blood cells no characteristic haemagglutinin pattern related to virulence could be discerned. Expression of surface haemagglutin(s) on *Aeromonas hydrophila* appears to be medium dependent; strains grown in liquid media demonstrated enhanced haemagglutination activity. Both virulent and avirulent strains had *in vitro* epithelial cell adhesive capabilities. Cell surface characteristics measured by agglutination in acriflavine and stability after boiling indicated that most virulent strains agglutinated in the presence of acriflavine, but not all sedimented after boiling. The ability of 10 selected strains of *A. hydrophila* to grow in normal pooled catfish serum was determined. Only 17% of the virulence variations can be explained by their sensitivity to serum.

Introduction

Motile *Aeromonas* spp. are a ubiquitous component of the aquatic environment (Nielson 1978), and are considered to be normal inhabitants of the intestinal tract of fish (Trust & Sparrow 1974; Ventura & Grizzle 1987). They are also considered to be significant bacterial pathogens of man (George, Nakata, Thompson & White 1985), fish and other cold-blooded animals (Shotts, Gaines, Martin & Prestwood 1972). *Aeromonas hydrophila* may produce a lesion in fish described as 'red-sore' disease, and is considered the primary bacterial aetiological agent of fish in the southeastern United States (Hazen, Fliermans, Hirch & Esch 1978; Plumb 1975). On one occasion in a North Carolina reservoir, more than 37 000 fish died over a 13-day period from 'red-sore' disease (Miller & Chapman 1976).

A prerequisite for the initiation of infection is the initial adherence of bacteria to host epithelial cells. Utilizing this fact, many investigators have attempted to associate *A. hydrophila* virulence to several systems such as adherence to rabbit brush borders (Levett & Daniel 1981), HEP-2 epithelial cells (Carrello, Silburn, Budden & Chang 1988) and human buccal cells (Atkinson & Trust 1980; Daily, Joseph, Duguid & Old 1980). Recently, Krovacek, Faris, Ahne & Mansson (1987) examined the adhesive potential of *A. hydrophila* and *Vibrio anguillarum* to fish cells. However, no description of the source of the isolates (fish or water) was provided nor was virulence potential for fish demonstrated. Previous reports indicate that there are variations in virulence among strains of *A. hydrophila* (Lallier, Mittal, Leblanc, Lalonde &

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Olivier 1981; Mittal, Lalonde, Leblanc, Olivier & Lallier 1980). In view of the absence of data on the adhesive capabilities of *A. hydrophila* strains virulent for fish, it was the present authors' objective to determine if variations in virulence can be attributed to surface characteristics, such as fimbriae or other surface molecules. In this study, the authors report the adherence, haemagglutination and cell surface characteristics of a number of *A. hydrophila* strains which are virulent for fish.

Materials and methods

Bacterial strains

Bacteria employed in this study were obtained from the stock collection at the Department of Medical Microbiology, University of Georgia, Athens, Georgia. Their sources are shown in Table 1. Cultures were identified by the scheme of Popoff & Veron (1976). Cultures were maintained at room temperature in tubes containing semi-solid maintenance media composed of 0·1 g tryptone, 0·1 g yeast extract and 4·0 g of agar/l of distilled water.

Virulence assay

The virulence assay was previously described by Hsu, Waltman & Shotts (1981). Briefly, channel catfish, *Ictalurus punctatus* Rafinesque, fingerlings weighing approximately 15–20 g were injected intramuscularly with a bacterial dose of 1 mg washed wet cells/100 g of fish at the lateral side below the dorsal fin. Ten fish were injected per bacterial strain, and a virulence index was estimated based on mortalities and grouped into eight levels:

Table 1. Sources of *Aeromonas* spp. examined

Isolate #	Species	Source	Location
09	*A. hydrophila*	Water	Montana
14	*A. hydrophila*	Fish	West Virginia
23	*A. hydrophila*	Fish	Florida
24	*A. sobria*	Water	Montana
36	*A. hydrophila*	Fish	Alabama
38	*A. sobria*	Water	Montana
49	*A. hydrophila*	Fish	North Carolina
53	*A. hydrophila*	Water	Montana
58	*A. hydrophila*	Fish	North Carolina
63	*A. hydrophila*	Fish	Texas
75	*A. hydrophila*	Unknown	Unknown
76	*A. hydrophila*	Fish	North Carolina
77	*A. hydrophila*	Water	North Carolina
79	*A. hydrophila*	Unknown	Unknown
84	*A. caviae*	ATCC 13137	
118	*A. sobria*	Water	Missouri
161	*A. hydrophila*	Water	Georgia
200	*A. hydrophila*	Fish	Alabama
248	*A. hydrophila*	Water	Georgia
266	*A. hydrophila*	Fish	Georgia
282	*A. hydrophila*	Human	Georgia

(7) more than eight fish killed in 1 day;
(6) more than eight fish killed in 2 days;
(5) more than eight fish killed in 3 days;
(4) more than eight fish killed in 1 week;
(3) five to eight fish killed in 1 week;
(2) less than five fish killed in 1 week;
(1) no fish killed in 1 week but at least two fish showed one or more of the pathological signs; and
(0) no fish were killed and no pathological signs were noted.

Fish were held up to 10 days after infection.

Electron microscopy

The presence of fimbriae on *A. hydrophila* grown in brain-heart infusion broth and agar was determined by negative staining using 2% phosphotungstic acid on Formvar coated carbon grids. The grids were examined with a JEOL 100S transmission electron microscope operating at 80 V.

Culture conditions

To test for mannose specific-binding activity, cultures were grown in brain-heart infusion (BHI) agar and broth (Gibco Laboratories, Grand Island, NY, USA). Agar and broth cultures were grown aerobically at 25°C. Serial subcultures were made on agar and in static broth cultures to enhance phase variation (two 24-h passages). Haemagglutinin activity and epithelial cell adherence was determined with washed cells that were serially passed in broth.

Yeast cell co-agglutination

The yeast cell co-agglutination assay was a modification of the procedure described by Mirelman, Altman & Eshdat (1980). The procedure was slightly modified by staining 1 ml of the inactivated yeast cell suspension with 30 μl of 0·8% trypan blue in phosphate buffered saline (PBS).

Haemagglutination assays

Guinea pig, chicken, cow and fish (channel catfish) red blood cells were collected by venipuncture with syringes containing heparin (final concentration 10 iu/ml) and stored at 4°C until required. Immediately before use, red blood cells were washed three times in PBS, pH 7·4. Channel catfish red blood cells were washed with PBS that had been previously adjusted to 250 Osm (Micro-osmometer Precision Systems Inc., Natick, Massachusetts, USA) and a 3% vol/vol suspension was prepared in PBS. Bacteria were grown statically in BHI broth with two 24-h aerobic subcultures before the assay. Slide haemagglutination assays were performed at 4 and 37°C. Equal volumes (25 μl) of blood suspension and washed bacterial suspensions were mixed on a slide with a depression and gently rocked by hand. A PBS control was always included. Strains were considered negative if haemagglutination had not occurred within 10 min.

To test for the ability of various carbohydrates to inhibit haemagglutination, 25 μl of a 1% w/v solution of the test carbohydrate was added to the washed bacterial suspension prior to the addition of the red blood cell suspension. The minimum haemagglutination dose (MHD) of bacteria was established and 5 × the MHD of bacteria was employed for the inhibition assay.

Reactions were compared to positive controls consisting of 25 μl bacteria, 25 μl PBS and 25 μl yeast suspension. Haemagglutination inhibition patterns were performed with both guinea pig and chicken red blood cells.

Epithelial cell adherence assay

Fathead minnow epithelial cells, a certified ATCC cell line, were employed. Cells were grown in a mixture of Leibowitz L-15 medium (Gibco) and minimal essential medium 199 (Gibco) at a 1:1 ratio and supplemented with 10% foetal bovine serum without antibiotics. Cells were seeded and grown to sub-confluency at room temperature. On the day of the assay, the cells were trypsinized (0·25%) and washed three times in tissue culture medium. Cells were then adjusted to approximately $1{\cdot}0 \times 10^5$ cells/ml with a haemocytometer. Viability was assayed by trypan blue (0·5%) exclusion. ^{32}P-labelled bacterial cells were obtained by inoculating 15 ml of a modified phosphorus deficient medium (Brenner, Steigerwalt, Falcao, Weaver & Fanning 1976), containing 15 uCi/ml of ^{32}P (orthophosphate) in 0·02 N HCl (New England Nuclear, Boston, Massachussetts, USA) with serially passaged strains of *A. hydrophila*. Labelling was allowed to proceed for 24 h at 30°C. To remove free label, bacterial cells were washed three times in tissue culture medium. The final bacterial concentration was adjusted to approximately $1{\cdot}0 \times 10^7$ Cfu/ml (OD, 660 nm 1·50 = $3{\cdot}0 \times 10^9$) by use of a spectrophotometer.

The modified medium was prepared from the following stock solutions. Stock A contained 40·0 g NH_4Cl, 60·0 g NaCl, 30·0 g KCl, 66·4 g TRIS and 2·28 g TRIS-HCl (pH 7.2)/l of distilled water. Stock B contained 11·0 g Na_2SO_4/100 ml distilled water. Stock C contained 11·0 g $MgCl_2{\cdot}6H_2O$/100 ml distilled water. The working medium was prepared by combining 50·0 ml of stock A with 1·0 ml of stocks B and C, and adjusting the final volume to one litre with distilled water. The addition of 2·0 ml (37 g/l distilled water) of BHI broth per litre was necessary to enhance growth.

For the adherence assay, 1·0 ml of ^{32}P-labelled bacterial suspension and 1·0 ml of epithelial cells were added to duplicate 50·0 ml polystyrene centrifuge tubes, tube contents mixed and tubes placed on a rotary shaker at 150 *g* for 1 h at 30°C. Unattached bacteria were then removed by layering 0·5 ml of the mixture onto a 7·0 cm cushion of 5·5% Ficoll-400 (Sigma Chemical Co., Saint Louis, Missouri, USA) in PBS.

The bottom of this cushion contained 0·3 ml of iso-osmotic Percoll (Sigma). Centrifugation was at 900 *g* for 3 min (IEC Model K, Damon/IEC Division, Needham Hts, Massachussetts, USA). Bacteria adhering to epithelial cells were easily removed at the Ficoll-Percoll interface by introducing a 22·5-cm pasteur pipette and withdrawing 0·5 ml. This fraction was then introduced into borosilicate scintillation vials containing 10·0 ml of 10 mM KH_2PO_4 solution (Clausen 1968). Radioactivity associated with the mixture was determined in a liquid scintillation counter (Beckman LSC 7000, Irvine, California, USA) with a pre-program setting of 0–397 KEV. The number of bacterial cells per epithelial cell was calculated from the ratio of radioactivity associated with the Ficoll-Percoll interface to the total radioactivity in the control sample (1·0 ml of bacteria added to 1·0 ml of tissue culture medium). Experiments were replicated three times and the mean adherence value was calculated.

Effects of acriflavine and boiling

Bacterial agglutination in acriflavine and the tendency of cells to sediment after boiling was assayed according to the procedure of Mittal *et al.* (1980).

Sensitivity to catfish serum

Fresh normal pooled catfish serum was used to test the sensitivity of selected isolates of *A. hydrophila*. The procedure used was as described by Mittal *et al.* (1980) with the exception that cultures were left in serum for 24 h before recording growth of inhibition.

Statistical methods

The data were analysed using point-biserial correlation analysis (pb), the Cramer correlation analysis (phi) or the Pearson correlation coefficient. The first method is used to correlate a dichotomous variable with a continuous variable. The second method measures the relationship between two dichotomous variables. The Pearson correlation coefficient associates continuous variables.

Results

Transmission electron microscopy demonstrated that both virulent and avirulent *Aeromonas* isolates were equally fimbriated (piliated) when grown in static broth cultures that had been serially passed (two 24-h passages) (Fig. 1A & B). Agar-grown cultures, on the other hand, were weakly or non-fimbriated (Fig. 1C & D).

Mannose-binding lectins are one of the mechanisms that mediate adherence of several types of bacteria to host epithelial cells (Ofek, Mirelman & Sharon 1977). To further evaluate the influence of cultural conditions on the expression of adhesins, a yeast cell co-agglutination assay was used to screen mannose-specific lectin activity (Mirelman *et al.* 1980). The results of the yeast co-agglutination assays indicated that only three of the 21 isolates examined did not show a mannose-specific lectin (Table 2) as demonstrated by inhibition of coagglutination with 1% mannose. Culturing in broth enhanced agglutination ability. Based on point biserial correlation analysis, no significant differences existed between virulence and ability to aggregate with yeast cells with either broth cultures (T = 0·56) or agar grown cultures (T = 1·0). Regression analysis also did not indicate a correlation between agglutination titre of either broth (R = 0·31) or agar (R = 0·22) grown strains and virulence.

Nineteen of the broth grown cultures agglutinated red blood cells from one or more species (Table 3). At the end of the initial incubation period at 4°C, the temperature was raised to 37°C. Haemagglutination was unaffected by the temperature increase. Different haemagglutination patterns could be discerned among the positive strains (Table 3). However, point biserial correlation analysis indicated no significant correlations between the ability to cause haemagglutination and ability to cause disease.

All the haemagglutinating strains studied formed a mannose sensitive haemagglutinin (MS-HA), which was detected with guinea pig red blood cells. Haemagglutination could also be inhibited with other carbohydrates (Table 4). Among the nineteen strains that caused haemagglutination, five carbohydrate inhibition patterns were observed. A majority of the strains (10 out of 19) were solely sensitive to mannuheptulose, mannose and α-methyl D-mannoside.

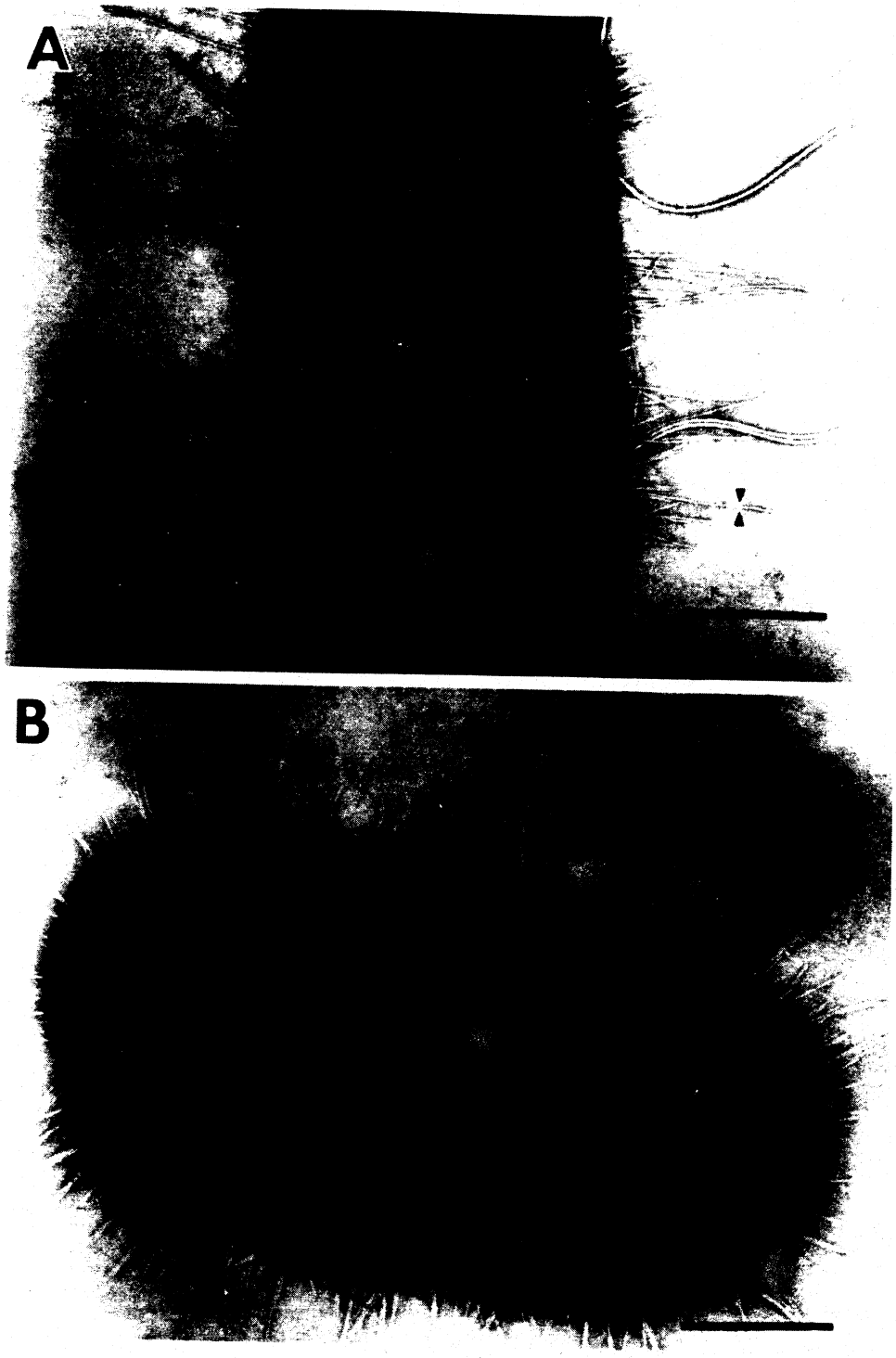
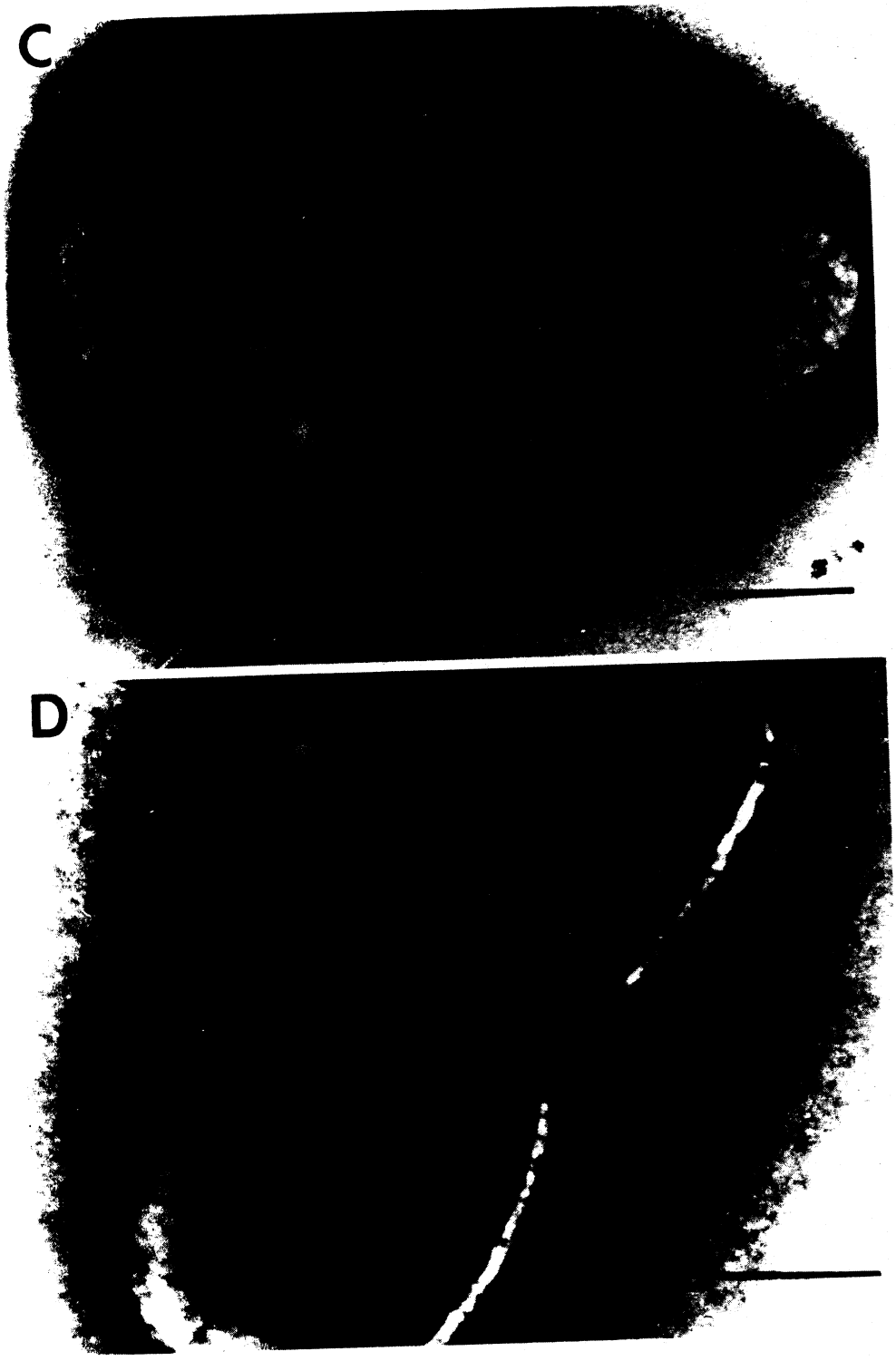


Figure 1. Electron micrographs of negatively stained broth- and agar-grown cultures: (A) strain #79, broth grown; (B) strain #118, broth grown; (C) strain #79, agar grown; and (D) strain #118, agar grown (bar = 0·5 μm).

These carbohydrates are structurally similar and it is possible that they were indicative of the same adhesin. A point biserial correlation analysis indicated that there was no statistical significance between virulence and carbohydrate inhibition patterns. The ability of strains to agglutinate chicken red blood cells correlated closely ($P < 0{\cdot}001$) with ability to agglutinate



guinea pig red blood cells, suggesting a common receptor. A positive correlation (phi correlation, $R = 0{\cdot}68^{+}$) was observed between L-fucose and galactose sensitivities. A negative correlation was evident between mannuheptulose and galactose ($R = -0{\cdot}54$) and between mannuheptulose and L-fucose ($R = -0{\cdot}79$).

Table 2. Virulence index and yeast co-agglutination of selected *Aeromonas* spp.

Isolate #	Virulence index	Yeast co-agglutination	
		Agar grown	Broth grown
09	7	+ (1:16)[1]	+ (1:64)
14	1	–	–
23	0	+ (1:64)	+ (1:128)
24	2	–	+ (1:128)
36	6	+ (1:32)	+ (1:128)
38	2	–	+ (1:4)
49	7	–	+ (1:64)
53	3	+ (1:16)	+ (1:64)
58	2	+ (1:8)	+ (1:128)
63	7	+ (1:18)	+ (1:128)
75	2	+ (1:4)	+ (1:8)
76	6	+ (1:64)	+ (1:128)
77	6	–	+ (1:128)
79	7	–	–
84	5	–	+ (1:256)
118	0	–	+ (1:64)
161	3	–	+ (1:64)
200	2	+ (1:16)	+ (1:64)
248	0	–	–
266	0	–	+ (1:4)
282	7	+ (1:16)	+ (1:64)

[1] Greatest dilution giving a positive agglutination response.

Table 3. Haemagglutination and yeast co-agglutination profile of *Aeromonas* spp.

Isolate #	Guinea pig	Chicken	Cow	Fish	Yeast[1]
#63(7), #200(2) #23(0), #24(2)	+	+	–	+	+
#77(6), #49(6) #36(6), #09(6) #76(6), #282(7) #53(3), #58(2) #38(2)	+	+	–	–	+
#79(7), #14(1) #248(0)	+	–	–	–	–
#161(3), #75(2)	–	–	–	–	+
#84(5), #118(5)	+	+	+	+	+
#266(0)	–	–	–	+	+

[1] Co-agglutination of *Aeromonas* spp. grown in broth with *Candida albicans*.

Table 4. Pattern of sugar inhibition of guinea pig haemagglutination by *Aeromonas* spp.

Number of isolates per group	Carbohydrate[1]			
	Mannitol	Galactose	Fucose	Mannuheptulose
4	+	−	−	+
1	−	−	−	−
11	−	−	−	+
1	−	−	+	−
1	−	+	+	−

[1] Haemagglutination of all strains was inhibited by mannose and α-methyl D-mannoside.

Selected *Aeromonas* strains with varied virulence were examined for *in vitro* epithelial cell adherence. Preliminary experiments established that the optimal conditions to achieve a 50% saturation was a 30°C incubation for 60 min using approximately 10^7 cfu/ml. Among the six isolates examined, the highest adherence value observed was with strain #118, an avirulent strain with an adherence value of 98 bacteria/cell. The other avirulent strain tested, #248, had an adherence value of only 22 bacteria/cell. Among the strains of intermediate virulence, strain #53 had an adherence value of 60 bacteria/cell and strain #161 a value of 75 bacteria/cell. Among the highly virulent strains examined, strains #63 and 79 had adherence values of 50 and 77 bacteria/cell, respectively. On the average, adherence values per experiment and within experiments ranged between ±10 bacteria/cell.

Twenty *Aeromonas* isolates were examined for their tendency to autoagglutinate and/or sediment after boiling. Four out of 20 strains auto-agglutinated in BHI broth after 24 h incubation at room temperature (Table 5). Three of them agglutinated in the presence of acriflavine and were unstable after boiling. One strain (#79) of 20 was only unstable after boiling. Five of the 20 strains that were stable after boiling did not agglutinate in acriflavine.

Table 5. Cell surface characteristics of *Aeromonas* spp. and their virulence

Isolate #	Agglutination in acriflavine	Precipitation after boiling	Auto-agglutination[2]
#63(7),[1] #49(6), #36(6) #84(5), #38(2)	+	+	−
#79(7)	−	+	−
#53(3), #14(1) #75(2), #58(2), #24(2)	−	−	−
#77(6), #9(6), #282(7) #200(2), #248(2)	+	−	−
#76(6), #161(3), #23(0)	+	+	+
#266(0)	−	+	+

[1] Parenthesis indicates virulence index: 0 = nonvirulent; 7 = highly virulent.
[2] Strains autoagglutinated in brain heart infusion broth after 24 h at 30°C.

Table 6. Sensitivity of *Aeromonas* spp. to normal channel catfish serum

Isolate #	Virulence index	Serum sensitivity (titre)
79	7	1:4[1]
77	6	1:4
49	6	1:4
09	6	R[2]
84	5	R
38	2	1:8
14	1	1:4
53	0	1:32
248	0	1:2
266	0	1:8

[1] Greatest dilution of serum producing inhibitory response.
[2] Resistant strain.

Ten *Aeromonas* isolates were examined for their ability to survive the bactericidal effects of normal catfish serum (Table 6). The results suggested a moderate (R = 0·41) positive relationship between serum sensitivity and virulence. Based on a coefficient of determination of R^2 = 0·165, approximately 17% of the variations in the virulence categories could be explained by the sensitivity to normal serum.

Discussion

The ability of *A. hydrophila* isolates to cause disease in fish appears to be related to multiple physiological and biochemical markers. Previous work in the authors' laboratory indicated a positive correlation between virulence and elastase or staphylolytic activity (Shotts, Hsu & Waltman 1985; Hsu *et al.* 1981). Likewise, virulence has been related to the ability of isolates o produce haemolysin(s) (Olivier, Lallier & Lariviere 1980; Allan & Stevenson 1981) or one of several proteases (Ljungh & Wadstrom 1982; Nieto & Ellis 1986; Thune, Graham, Riddle & Amborski 1982; Leung & Stevenson 1988). A number of physiological processes related to cell surface characteristics have been associated with virulence in a variety of bacterial species. For example, the ability of a bacterial cell to adhere to the surface of a host cell is a virulence associated property in a number of pathogens. The purpose of the current project was to determine if the ability of *A. hydrophila* isolates to actively cause disease in fish could be related to any of several characteristics associated with the cell surface. However, it should be pointed out that the protocol for assessing virulence in the current study (i.e. intramuscular injection) would not assess directly the initial attachment to host tissue, a potentially integral part of the infection process in non-injured fish (Munro 1982). The decision to employ this route of entry was based on the need to control accurately the infective dose, and the effectiveness of the mucus on the surface of the fish as a barrier to bacterial infection. Further, recent work has indicated that skin injuries from fin spines are likely to occur when channel catfish are crowded, thus allowing entry of microorganisms. Ventura & Grizzle (1987) demonstrated that abraded skin was a more effective portal of entry than intact skin or the alimentary tract.

The current study established that the formation of fimbriae (pili) by *Aeromonas* was dependent on cultural conditions, with serial passage in broth cultures enhancing the extent of fimbriation. Fimbriae were observed with both virulent and avirulent strains, which is not in agreement with Lallier & Daignault (1984), who correlated the presence of fimbriae with pathogenicity toward fish. Kirov, Rees, Wellock, Goldsmid & Vangalen (1986) observed that clinical (enterotoxigenic) strains were generally poorly or non-fimbriated, whereas environmental (non-enterotoxigenic) isolates possessed fimbriae. Trust, Courtice & Atkinson (1980) found that only one of 11 strains of *A. hydrophila* formed fimbriae. It is important to note that these other investigations used solid media, which in the present authors' hands retards fimbriation (Table 2).

The results of the current investigation emphasize the complex nature of cell surface associated properties in *Aeromonas*. No pattern of relatedness was evident among the various analyses, nor could the majority of surface characteristics be related directly to virulence in fish. For example, the carbohydrate inhibition studies (Table 4) indicated that while haemagglutination was blocked in all positive strains by mannose and α-methyl D-mannoside, there was substantial diversity among the isolates in their response to other sugars. Five subgroups were identified. Comparison of these results with those from the yeast co-agglutination assay indicated an additional level of complexity. The co-agglutination test was employed as a means of assessing the presence of mannose/mannan sensitive adhesins on the surface of the bacterial cell, and it would be expected that the results of this assay would be consistent with the inhibition of haemagglutination by mannose (Table 3). However, two isolates (74 and 161) were identified as haemagglutination-negative, but yeast agglutination-positive. Conversely, three strains (14, 79 and 248) that were yeast agglutination-negative, were haemagglutination-positive and their attachment to erythrocytes was inhibited by mannose. Interestingly, all of this second group of isolates fell within a single haemagglutination subgroup that only agglutinated guinea pig erythrocytes. This suggests that aeromonads had adhesins specific for a moiety present on guinea pig erythrocytes but absent from other types of red blood cells. These results also imply that the yeast co-agglutination assay may not directly measure mannose-mediated attachment, since mannose did inhibit haemagglutination of the three strains negative in the yeast assay. Neither haemagglutination nor yeast co-agglutination appear directly correlated with virulence.

Six tested strains (53, 63, 79, 118, 161 and 248) adhered at varying degrees to fathead minnow epithelial cells. No relationship with virulence was evident since both virulent and avirulent strains showed attachment. No correlation was apparent between adherence in this *in vitro* cell line assay and attachment in either the haemagglutination or yeast co-agglutination assays, but both positive and negative strains from the latter two assays were capable of adhering to the fathead minnow cells. This suggests that the mechanism of attachment to the cell line is different from those involved with either haemagglutination or yeast co-agglutination. Using two fish cell lines, Krovacek *et al.* (1987) examined the adhesive potential of three fish and two human isolates of *A. hydrophila*. Unlike the current study, they observed that only one isolate adhered strongly, having an average attachment rate of over 100 bacteria/cell.

Studying a variety of characteristics, Mittal *et al.* (1980) concluded that virulent strains of *Aeromonas* could be differentiated by cell surface characteristics and serogrouping. They observed that highly virulent strains did not agglutinate in acriflavine, were resistant to normal mammalian serum, and sedimented after boiling. Lallier & Daigernault (1981) also reported that lack of agglutination in acriflavine was highly correlated with fish pathogenicity. Janda, Oshiro, Abbott & Duffey (1987) observed that among 10 acriflavine-negative strains, nine

were lethal to mice. However, seven out of 29 acriflavine-positive strains were also lethal for mice. In the current study, a majority of the highly virulent isolates clustered in two groups (Table 5). The first group were strains that agglutinated in the presence of acriflavine and precipitated after boiling, but did not autoagglutinate. The second group were strains that agglutinated in the presence of acriflavine, but did not autoagglutinate or precipitate after boiling. The majority of the strains from the current study that fit the profile that Mittal *et al.* (1980) considered indicative of virulence were only weakly to intermediately virulent. Conversely, the majority of strongly virulent isolates in the current investigation would have been predicted to be non-virulent using the criteria of Mittal *et al.* (1980). Janda *et al.* (1987) reported that agglutination after boiling was a virulence-associated marker for motile *Aeromonas*. A majority of the highly virulent strains (virulence index ≥ 5) in the current study precipitated after boiling (Table 5). However, 30% (three out of nine) of the highly virulent isolates were negative for this attribute, indicating that, at least by itself, this marker would be of limited usefulness for assessing virulence in *Aeromonas*.

The significance of autoagglutination to virulence in fish is not known. Autoagglutination was apparent with four out of 20 isolates in the current study, with these strains including both virulent and avirulent strains. Mittal *et al.* (1980) did not observe autoagglutinating strains. Janda *et al.* (1987) reported that nine out of 79 isolates of *Aeromonas* spp. had the ability to autoagglutinate. Namdari & Bottone (1988) concluded that autoagglutination was a 'suicidal phenomenon' observed only when avirulent clinical isolates were cultured with dextrose. However, in the current study one of the autoagglutinating strains was strongly virulent to fish. Likewise, a number of the avirulent and weakly virulent strains did not autoagglutinate. Further research is needed to assess the importance of autoagglutination as a virulence-associated marker.

Evaluation of 10 *Aeromonas* strains to normal catfish serum suggested a positive relationship between this characteristic and virulence in that two of the highly virulent, but none of the weakly or non-virulent, strains were strongly resistant. However, the correlation was much weaker when the quantitative response to dilution was considered; the difference between virulent and avirulent strains was less clear cut. Mittal *et al.* (1980) observed that only virulent nd weakly virulent strains were resistant to normal mammalian serum.

In summary, the results of the current study indicate that surface related characteristics in otile *Aeromonas* species are complex, and there may be an array of adhesins involved in the tached of the bacterium to various tissues. There appears to be substantial diversity among *eromonas* isolates as evidenced by the range of subgroups evident with each of the various gglutination assays. Further, the current study demonstrates effectively that cultural conditions can dramatically influence the expression of potential surface-related characteristics. None of these could be unequivocally related to pathogenicity based on the method used for assessing the relative virulence of the isolates. However, there was a degree of agreement observed between virulence and specific analyses such as the clustering of highly virulent strains in regard to combinations of characteristics (i.e. agglutination after exposure to boiling or acriflavine). This suggests that targeting these areas for future research efforts could lead to a better understanding of the role that cell surface characteristics in *A. hydrophila* and related motile *Aeromonas* spp. play in initiation and maintenance of disease states in fish.

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